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Plain polystyrene microplastics reduce the toxic effects of ZnO particles on marine microalgae *Dunaliella salina*.

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ABSTRACT

A critical literature survey on marine ecotoxicology reveals a lack of comprehensive studies to assess the impact of microplastics on the toxicity of engineered nanomaterials at environmentally relevant doses. Though ZnO and microplastics both are well known to be marine pollutants, the combined toxicity of ZnO particles with plain polystyrene (PS) microplastics are yet to be studied. Preliminary characterization of ZnO particles included examining particle size, morphology, and surface area. The amount of nominal and dissolved ions in the suspensions containing nano-sized ZnO particles was determined. The toxicity of bulk and nano-sized ZnO particles in combination with plain PS microplastics at low concentration (1 mg/L) was assessed towards marine algae *Dunaliella salina* at three exposure concentrations 1.22, 12.28 and 122.88 μM under UV-A and dark exposure conditions. As expected, a dose-dependent increment in the toxicity, ROS (extracellular & intracellular) generation and lipid peroxidation were noted for both bulk and nano-sized ZnO particles. The harmful effects of bulk and nano-sized ZnO particles were considerably reduced in the presence of plain PS microplastics. This study opens up new dimensions regarding the positive impact of microplastics at low concentration, where they lessen the toxic effects of co-pollutants in the marine ecosystem.

1. Introduction

Amongst the well-known engineered nanomaterials, metal oxide nanoparticles are quite extensively used in various applications [1]. From this group, nano-sized ZnO particles find their place in a wide variety of consumer products including paints, sunscreens and coatings [2]. Worldwide production of ZnO nanoparticles is said to vary between 5.5–28,000 t/year [3]. So, the release of these particles from the commercial products into the marine environment through various routes like surface runoff and wastewater discharge seems inevitable [4].

Microalgae are known as primary producers of the food web and are regularly used as a bioindicator for marine pollutants [5]. Pent et al. [6] assessed the harmful effects of nano-sized ZnO particles using two different particle sizes, 6.3 and 15.7 nm, and found that the smaller particle exhibited increased toxicity. In another report, Lee and An indicated that nano-sized ZnO particles led to the destabilization of the cell membrane [7]. The genotoxic and cytotoxic effects of ZnO nanoparticles against *Dunaliella tertiolecta* was documented [8], and the harmful effects of bulk and nano-sized ZnO particles on the green alga *Tetraselmis* and *Phaeodactylum* sp. were recently assessed by another

research group [9]. There are comprehensive reviews underlining the toxic effects of nano-sized ZnO particles on different seawater species including marine algae [10,11]. A prior study noted that both particles and ions are expected to contribute to the toxicity of nano-sized ZnO [12].

Plastic fragments with dimensions < 5 mm are known as microplastics and they pose a severe risk to marine ecosystems [13]. From a recent estimate, there are around 51.2×10^{12} microplastics particles polluting the seawater globally [14]. Microplastics can exert toxic effects on microalgae, which involve growth reduction and generation of ROS [15]. The photosynthesis and growth of microalgae, *Dunaliella* sp. was adversely affected by the treatment with various sizes 0.05, 0.5 and 6 μM of polystyrene particles over 72 h [16]. In another report [17], the toxic impacts of microplastics of two different sizes (1 μm and 1 mm) on *Skeletonema costatum*, marine phytoplankton, were examined. The microplastics (average diameter 1 μm) caused significant growth inhibition while the plastic debris (average diameter 1 mm) did not show any effects.

Recent studies confirm that ZnO nanoparticles can severely affect marine algae [12,18–20]. There are also reports clearly showing the

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harmful effects of polystyrene microplastics toward marine algae [21,22]. It is understood that in the marine ecosystem the pollutants co-exist and their interactive effects on the organisms cannot be overlooked. A recent study highlighted that microplastics could exacerbate the toxicity of gold nanoparticles to the marine microalgae *Tetraselmis chuii* [23]. Another study reported growth inhibitory effects of copper nanoparticles with microplastic on microalgae *Skeletonema costatum* [24]. We have also studied the mixture effects of differently functionalized microplastics and P25 TiO₂ in marine algae *Chlorella* sp [25]. Thus, the combined effects of engineered nanoparticles with microplastics toward marine algae need to be assessed critically. Though nano-sized ZnO particles and the microplastics are well established marine pollutants, no prior report exists regarding their combined effects in the marine algae.

To fill this critical gap, the current study hypothesizes that the plain PS microplastics would influence the impacts of bulk and nano-sized ZnO particles in marine microalgae. The influence of uncharged plain microplastics at a concentration (1 mg/ L) on the toxic effects of bulk and nano-sized ZnO particles (1.22, 12.28 and 122.88 μ M) with UV-A and dark exposure conditions was evaluated by cell viability, abiotic and intracellular ROS generation, lipid peroxidation, dissolution and zinc salt toxicity analysis.

2. Materials and methods

2.1. Materials

In this study, two different sizes of uncoated Zinc oxide (ZnO) particles were used. They are denoted as nano (< 50 nm with > 97 % purity) and bulk (< 5 μ m with 99.9 % purity) sized ZnO particles. These particles and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich, USA. Other chemicals used in the study such as MTT (3-[4, 5-dimethylthiazol- 2-yl]-2, 5-diphenyltetrazolium bromide), DMSO (Dimethyl sulfoxide), TBA (Thiobarbituric acid) and TCA (Trichloroacetic acid) were acquired from Hi-Media Pvt. Ltd, Mumbai, India. Corpuscular Inc. is the company from where the main component of the study, plain PS microplastics of 2.5 % w/v stock concentration with $\rho = 1.06 \text{ g/cm}^3$ and specific size of 6 μ m was obtained from the USA.

2.1.1. Algal culture maintenance

The marine algae used in our study *Dunaliella salina* was procured from CMFRI (Tamil Nadu, India). The algal cell culture was allowed to grow at $23 \pm 2^\circ\text{C}$ (light/dark cycle of 16 h/8 h, under fluorescent white light with $40.5 \mu\text{mol m}^{-2}\text{s}^{-1}$ photon density, along with the necessary nutrients (Supplementary Table S1).

2.1.2. Preparation of ZnO particle suspension and microplastics

The fresh stock solution of bulk and nano-sized ZnO particles (100 mg/L) were prepared in Milli-Q water. Approximately 30 ml of Milli-Q water was added separately to 3 mg of bulk and nano-sized ZnO particles and subsequently sonicated at 20 kHz & 130 kHz using Sonics, vibra cell (USA) for 20 min to ensure uniform dispersion. All the test samples were treated with 1 mg/ L microplastics dispersed in a suspension of 8.93×10^9 particles/mL.

2.2. Preliminary characterization of ZnO particles and polystyrene microplastics

The structural morphology of bulk and nano-sized ZnO particles were characterized by High-Resolution Transmission Electron Microscopy (HRTEM, FEI TecnaiG2 T20 S-twin). Before analysis, the samples were sonicated and mounted on a copper grid. The particle size of plain PS microplastics was also examined under a scanning electron microscope (SEM), Model S400, HITACHI, Japan. Herein, plain PS microplastic suspensions (1 mg/ L) were prepared and coated on a thin

glass slide and dried [25]. The surface area specific to bulk and nano-sized ZnO particles were analysed by the BET method, (Brunauer Emmett Teller) Micrometrics, Tris-tar II 3020 from the USA.

2.2.1. Determination of nominal and dissolved ions

The amount of elemental Zn was quantified from nano-sized ZnO particles dispersed in an artificial seawater medium [26]. The quantity of elemental Zn present in nano-sized ZnO particles was evaluated for all working concentrations (1.22, 12.28 and 122.88 μ M) at a zeroth hour in artificial seawater (ASW). The above suspension was centrifuged twice at 13,000 rpm (4°C , 30 min) and the collected supernatant filtered using a 0.1 μ m syringe filter subsequently with 3 kDa. The quantification of elemental Zn contained in the samples was analyzed by AAS using AAAnalyst400 (PerkinElmer, India) at a wavelength of 345 nm. The release of Zn^{2+} ions from nano-sized zinc oxide particle of concentration 122.88 μ M were measured in the presence and absence of microplastics (1 mg/ L) at 0, 24 and 72 h time intervals under UV-A and dark environmental conditions.

2.2.2. Stability, surface charge and sedimentation of ZnO particles in the test medium

The effective diameter of bulk and nano-sized ZnO particles, as well as the surface charge of plain PS microplastics and algae, *Dunaliella* sp. were measured using DLS and zeta analyser (90 Plus Particle Size Analyzer with zeta facility) Brookhaven Instruments Corp., USA. To measure the effective diameter at different time intervals of 0 and 24 h under UV-A and dark environmental conditions, the bulk and nano-sized ZnO particles with varying concentrations (1.22, 12.28 and 122.88 μ M) were sonicated in Milli-Q water and further dispersed in artificial seawater.

The absorbance and sedimentation of bulk and nano-sized ZnO particles were analysed using a UV-vis spectrophotometer (Model 2201, Systronics, India) at 364 nm. Herein, the bulk and nano-sized ZnO particles (122.88 μ M) dispersed in ASW were taken at various intervals of time 0, 24, 48 and 72 h under UV-A and dark environmental conditions.

2.3. Experimental setup

The toxicity of bulk and nano-sized ZnO particles in combination with plain PS microplastics towards the algae of marine water i.e., *Dunaliella salina* was investigated. In this, *Dunaliella salina* was cultured using a filtered and sterilized natural seawater medium [25]. The analysis of the toxicity of bulk and nano-sized ZnO particles was performed, based on OECD recommendations (2004). The algal cells were allowed to grow until the exponential stage which was then subjected to centrifugation at 7000 rpm (10 min, 4°C). The supernatant was removed and the obtained pellet was resuspended in artificial seawater (ASW) and adjusted to 0.5 OD [27]. Throughout, the toxicity experiments were carried out in ASW (Supplementary Table S3) under UV-A and dark environment for 72 h incubation.

2.3.1. Cell viability assessment

2.3.1.1. ZnO particles and microplastics. Based on the previous study, the EC50 values for nano-sized ZnO particles for *Dunaliella tertiolecta* were reported to be 2 mg/L [12]. The algal cells with 0.5 OD was mixed with different concentrations of bulk and nano-sized ZnO particles (1.22, 12.28 and 122.88 μ M). The combined toxicity evaluation of the selected concentration of bulk and nano-sized ZnO particles with plain PS microplastics (1 mg/ L) was also performed. The interactions were continued for 72 h under UV-A and dark conditions. The algal suspension was used as a control without bulk and nano-sized ZnO particles. MTT assay was carried out to determine cell viability after 72 h. For cell viability assessment, 500 μ l of control and nominal concentrations of bulk and nano-sized ZnO particles treated with algal samples were added and incubated in the dark for 4 h along

with 20 μ l of MTT dye. The suspension was centrifuged at 8000 rpm for 8 min. The supernatant was discarded and 500 μ l of artificial seawater was added to wash the pellet again by centrifugation. After adding 200 μ l of DMSO to the pellet after washing, (ELISA microplate reader), Biotek, Powerwave XS2 was used to measure the absorbance at 570 nm. In terms of control cells, the percentage loss in cell viability of treated cells was measured.

2.3.1.2. Zinc salt and microplastics combination. Similarly to what was discussed earlier, the cell viability assessment of *Dunaliella salina* treated with zinc salt and microplastics were also performed to determine the viability of algal cells. The algal cell suspension interacted with a 55.3 μ M concentration of zinc sulphate in the presence and absence of plain PS microplastics (1 mg/ L) under two exposure conditions for 72 h, using the same protocol as in the previous section. As the maximum dissolution of Zn^{2+} was 55.3 μ M from nano-sized ZnO particle by the end of 24 h, the same concentration was selected to assess the toxicity of Zn ions released from zinc salt towards *Dunaliella salina*.

2.4. Evaluation of oxidative stress

2.4.1. Extracellular production of ROS

Extracellular reactive oxygen species (ROS) production was estimated using two NP fluorescent probes: 2',7'- dichlorodihydrofluorescein (H_2DCF), which reacts with hydroxyl radical (OH^\bullet), and oxygen and nitrogen species, which is specific to 3'-(p-hydroxyphenyl fluorescein) from HPF, Life Technologies [28]. The algal cells at different concentrations with bulk and nano-sized ZnO particles (1.22, 12.28 and 122.88 μ M) and plain PS microplastics (1 mg/ L) interacted with HPF for 72 h as H_2DCF decomposes under illumination (UVA). Briefly, deacetylation of H_2DCF -DA was performed by dissolving H_2DCF -DA in 1.3 mM ethanol, further incubated in the dark for 30 min with 4 ml of NaOH (0.01 N). The reaction was terminated by the addition of a 20 ml sodium phosphate buffer (25 mM) at pH 7.4 to give a solution of H_2DCF (52 μ M). The mixture was then cooled and stored in the dark for later use. Further, H_2DCF and NP suspension of 100 μ l each were mixed and incubated in room temperature for 45 min in 96-well black microplate. A microplate fluorometer was used to measure fluorescence at 485 nm excitation and 527 nm emission wavelength at the end of incubation using (Fluoroskan Ascent FL), Thermo Labsystems, Helsinki, Finland. The fluorescent dye was used to measure the extracellular ROS rate which is represented in relative fluorescence units (RFU).

2.4.2. Determination of ROS (Intracellular)

A cell-permeable dye, 2',7'- dichlorofluorescein diacetate, was used to detect intracellular reactive oxygen species [29]. For cellular ROS staining, 49.2 μ l DCFH-DA was applied to 5 mL of control and the algal suspension treated with bulk and nano-sized ZnO particles (1.22, 12.28 and 122.88 μ M) and plain PS microplastics (1 mg/ L), followed by 30 min of dark incubation. Individual and combined toxic effects of treated samples were analysed by measuring dichlorofluorescence using a spectrofluorometer (SL174, ELICO) India, at an excitation wavelength of 485 nm, with emission at 530 nm. The percentage of intracellular ROS production was determined and compared with the control cells.

2.4.3. Determination of lipid peroxidation

The LPO assay was conducted to determine the production of malondialdehyde (MDA), which is mainly due to the stress caused in the algal cells [30]. For the treatment of individual bulk and nano-sized ZnO particles (1.22, 12.28 and 122.88 μ M), plain PS microplastics (1 mg/ L) and their combinations, the LPO generated by the cells were calculated. After 3 days, these samples were centrifuged at 7000 rpm (10 min, 4 $^\circ$ C). More specifically, the cell pellet was added to the mixture containing 2 ml of TBA and TCA of concentrations of 0.25 % (w/v) and 10 % (w/v), respectively. The above mixture was incubated for 30 min at 95 $^\circ$ C in a water bath. The heated samples were then cooled and centrifuged (7000 rpm, 10 min.). Finally, the absorbance of the supernatant was recorded at two wavelengths 532 & 600 nm using a UV-vis spectrophotometer (HITACHI, Model U2910) from Japan. The percentage of LPO produced with respect to control cells was estimated.

2.5. Statistical analysis

Triplicates were performed for all the experiments to calculate the standard deviation. The significant difference between the control with pristine bulk and nano-sized ZnO particles and the differences between the treatment groups, bulk and nano-sized ZnO particles both in the presence and absence of plain PS microplastics under UV-A and dark conditions were measured using two-way ANOVA (Bonferroni post-test) code, and Graph Pad Prism. In all cases, the statistical significance has been accepted at $p < 0.05$ level.

3. Results

3.1. Preliminary characterization of zinc oxide particles and microplastics

The size of nano-sized ZnO particles was around 21 nm \pm 4.5 nm (Fig. 1a), with spherical morphology. The effective diameter of nano-

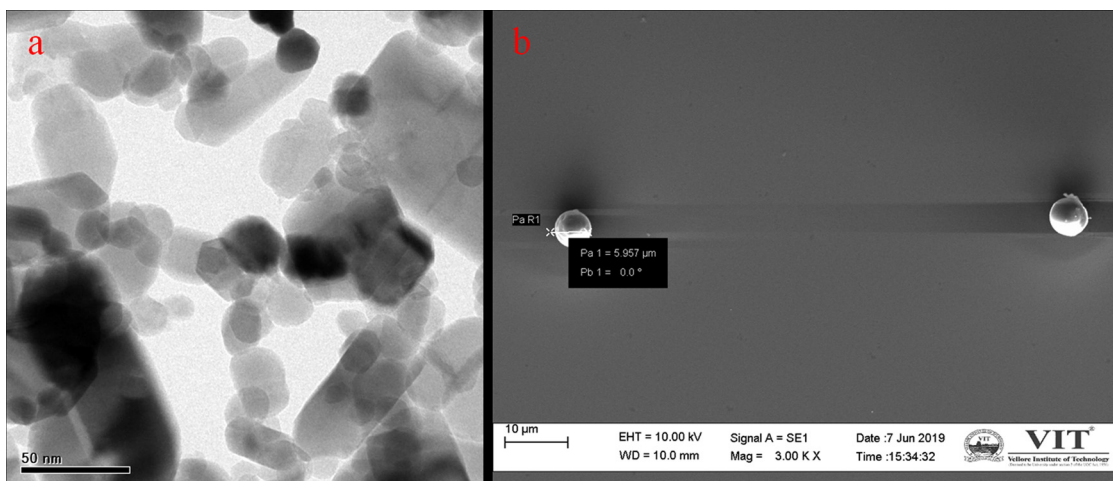


Fig. 1. (a) Transmission electron microscopic image of nano-sized ZnO particle and (b) Scanning electron microscopic image of plain PS microplastic.

sized ZnO suspended in Milli-Q water was found to be 251.65 ± 9.05 nm (polydispersity index of 0.24 ± 0.010) and 975.18 ± 85.89 nm for bulk-sized ZnO (polydispersity index of 0.30 ± 0.01). The SEM image of plain PS microplastics displayed spherically shaped particles (Fig. 1b) having a size of $6 \mu\text{m}$. The surface area of the bulk and nano-sized ZnO particles was found to be $21.66 \text{ m}^2/\text{g}$ and $6.47 \text{ m}^2/\text{g}$ respectively.

3.2. Determination of nominal and dissolved ions

The amount of elemental Zn present in nano-sized ZnO suspensions (1.22, 12.28 and $122.88 \mu\text{M}$) was found to be 0.98, 8.47, $83.55 \mu\text{M}$. After the addition of plain PS microplastics (1 mg/L), whilst the elemental Zn present in the nano-sized ZnO suspensions (1.22, 12.28, $122.88 \mu\text{M}$) was found to be 0.61, 8.35, $76.18 \mu\text{M}$.

The release of Zn^{2+} ions from nano-sized ZnO particles in artificial seawater medium was quantified at 0, 24 and 72 h intervals and shown in Fig. S5, Supplementary information). In the presence of plain PS microplastics, the amount of ions released at all time intervals under both the lighting conditions was noted in Fig. S5, Supplementary information. The amount of Zn^{2+} ions released at 0 h was found to be $28.2 \mu\text{M}$. At 24 h, the amount of ions released was about $54 \mu\text{M}$ under both UV-A and dark conditions. After a 72 h time interval, the Zn^{2+} ions released was about $41.7 \mu\text{M}$ under UV-A conditions and $38 \mu\text{M}$ under dark conditions. Similarly, the Zn^{2+} ions released after the respective exposure periods were statistically significant ($p < 0.05$) whereas for UV-A and the dark environment, the difference was found to be statistically insignificant.

3.3. Stability of ZnO particles in test medium

The effective diameter analysis of bulk and nano-sized ZnO particles dispersed in ASW was performed at two different time intervals: 0th h

and 24th h (Fig. 2). Initially, at 0th h, the effective diameter of bulk and nano-sized ZnO at 1.22, 12.28 and $122.88 \mu\text{M}$ concentrations were within the sub-micron size range. After 24 h, the size of bulk and nano-sized ZnO particles increased to micron size under both UV-A and dark conditions. The size of both ZnO particles types increased with increasing concentration. The difference in effective diameter at 0th and 24th h was statistically significant ($p < 0.05$) for 12.28 and $122.88 \mu\text{M}$ concentrations, in the case of nano-sized ZnO under both UV-A and dark conditions and was statistically significant ($p < 0.05$) only at the highest concentration of $122.88 \mu\text{M}$ for bulk-sized ZnO particles under both exposure conditions (Fig. 2).

The surface charge of bulk and nano-sized ZnO particles dispersed in ASW were found to be positive with potentials of $7.3 \pm 2.6 \text{ mV}$ and $10.49 \pm 2.48 \text{ mV}$, respectively. The surface charge of *Dunaliella* sp. was reported to be negative with a potential value of $-4.5 \pm 1.2 \text{ mV}$. The sedimentation of bulk and nano-sized ZnO particles at $122.88 \mu\text{M}$ concentration in ASW is represented in Figs. S1 and S2, Supplementary information. The reduction in absorbance of bulk and nano-sized ZnO particles was observed after 24 h. This decrease was statistically significant ($p < 0.05$) for both the types of particles under UV-A and dark conditions.

3.4. Cell viability assessment

3.4.1. Zinc oxide particles and microplastics

The cytotoxic effect of bulk and nano-sized ZnO particles individually and in combination with plain PS microplastics was calculated. A decrease in cell viability was noticed with increasing concentrations of bulk and nano-sized ZnO particles under UV-A illumination and dark conditions. This decreased cell viability was statistically significant ($p < 0.05$) compared to that of the control irrespective of test concentrations, which is graphically represented in (Fig. 3). The decreased toxicity in the case of bulk size particles

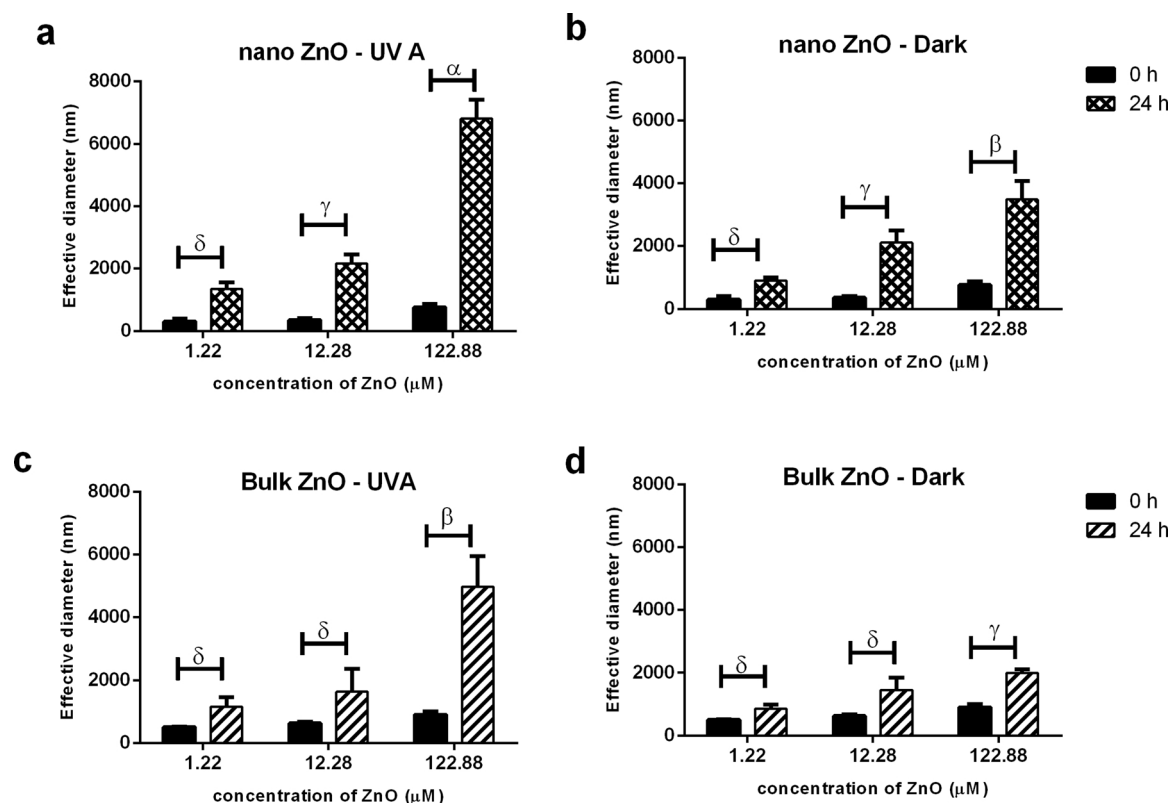


Fig. 2. Effective diameter of (a and b) nano-sized ZnO particle under UV-A and dark conditions, and of (c and d) bulk ZnO particle under UV-A and dark conditions respectively in ASW medium ($n = 3$; $\alpha = p < 0.001$, $\beta = p < 0.01$, $\gamma = p < 0.05$ and δ (ns) denoted the significance between time 0 h and 24 h).

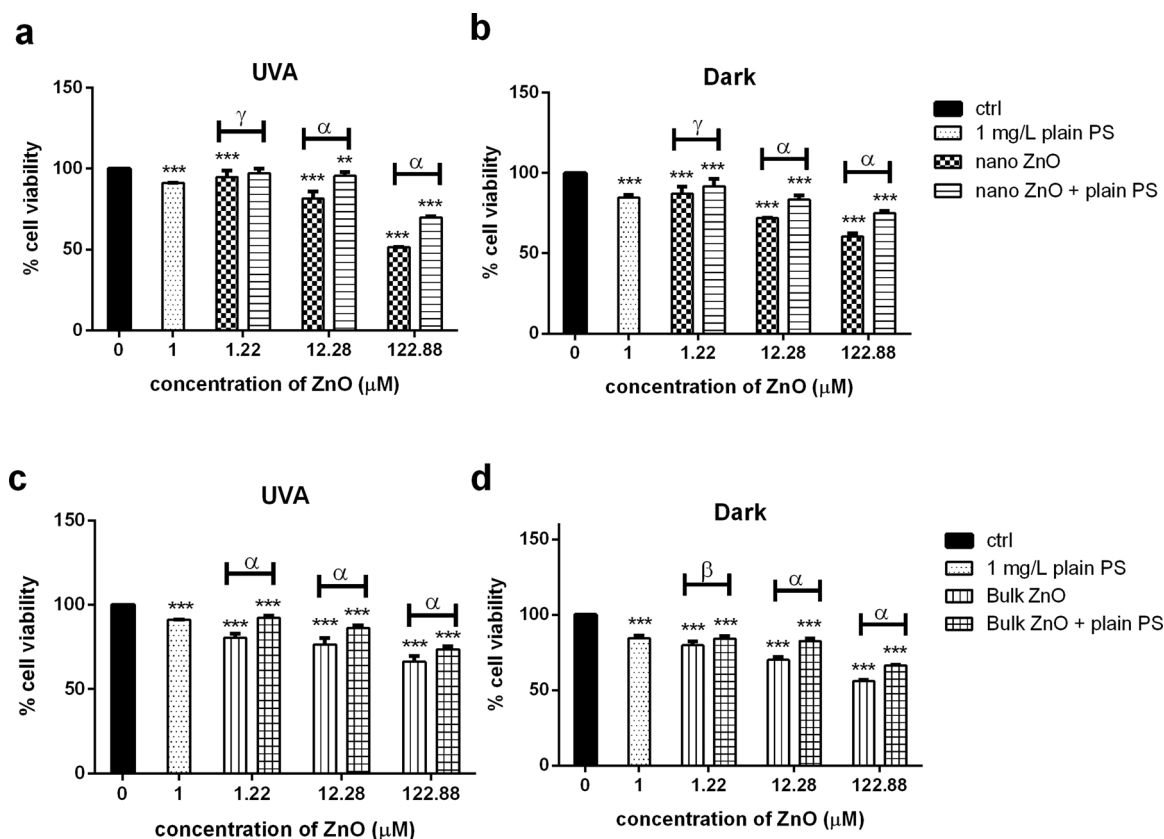


Fig. 3. Percentage cell viability for (a and b) nano-sized ZnO particle and (c and d) bulk ZnO particle treated algae in the absence and presence of plain PS microplastics under UV-A and dark conditions ($n = 3$). * denoted the significance between the individual nano ZnO and bulk ZnO particle with control algae ($***p < 0.001$ and $**p < 0.01$). α , β , γ and δ denoted significance between the nano ZnO and bulk ZnO particle in the presence and absence of microplastics ($\alpha = p < 0.001$, $\beta = p < 0.01$, $\gamma = p < 0.05$ and δ (ns)).

compared to nano-sized ZnO under UV-A conditions proves nano size-specific effects. The toxic effects were considerably enhanced under UV-A illumination for both particle types compared to dark conditions.

In the presence of 1 mg/L of plain PS microplastics, cell viability was enhanced for both bulk and nano-sized ZnO particles for all test concentrations. This increase was statistically significant ($p < 0.05$) for the combination of bulk and nano-sized ZnO particles with plain PS microplastics, in comparison with the respective concentrations of pristine bulk and nano-sized ZnO particles. A similar trend was also observed upon the addition of plain PS microplastics to bulk-sized ZnO under both the conditions. The cell viability was observed to be more enhanced under UV-A condition compared to the dark condition ($p < 0.05$) at all the test concentrations.

3.4.2. Zinc salt and microplastics

Cell viability assessment for the treatment of zinc sulphate individually and in combination with plain PS microplastics is represented in Fig. S6, Supplementary information. A decrease in the cell viability was noted at a concentration of 55.3 μM of zinc sulphate under UV-A and dark conditions, and this decrease in cell viability with respect to control under both conditions was statistically significant ($p < 0.05$). On the other hand, the cell viability is considerably increased by the addition of 1 mg/L plain PS microplastics. This increase was found to be statistically significant ($p < 0.05$) for the combination of zinc sulphate with plain PS microplastics in comparison with pristine zinc sulphate under both conditions.

3.5. Determination of oxidative stress

3.5.1. Abiotic ROS generation

The percentage of extracellular ROS produced by bulk and nano-sized ZnO particles individually and in combination with plain PS microplastics is graphically represented in (Fig. 4). The increase in extracellular ROS production is well correlated with the concentrations of bulk and nano-sized ZnO particles, both under UV-A and dark conditions. This increase in extracellular ROS production was statistically significant ($p < 0.05$) compared to that of the control, irrespective of test concentrations. No specific differences were observed between bulk and nano-sized ZnO particles, but ROS generation was more pronounced under UV-A conditions compared to that in dark conditions.

In mixtures with plain PS microplastics, a decline in the extracellular reactive oxygen species production was observed in bulk and nano-sized ZnO particles for all test concentrations. The reduction in extracellular reactive oxygen species production was noticed to be enhanced under UV-A as opposed to under dark conditions ($p < 0.05$) for all test concentrations.

3.5.2. Intracellular reactive oxygen species production

The percentage of intracellular reactive oxygen species produced by bulk and nano-sized ZnO particles individually and in combination with plain PS microplastics is graphically represented in (Fig. 5). A concentration-dependent increment in the intracellular ROS production was noticed with increasing concentrations of bulk and nano-sized ZnO particles both under UV-A and dark conditions. This increased generation of intracellular ROS was statistically significant ($p < 0.05$) with respect to the control for all test concentrations.

With the addition of plain PS microplastics, a decline in the

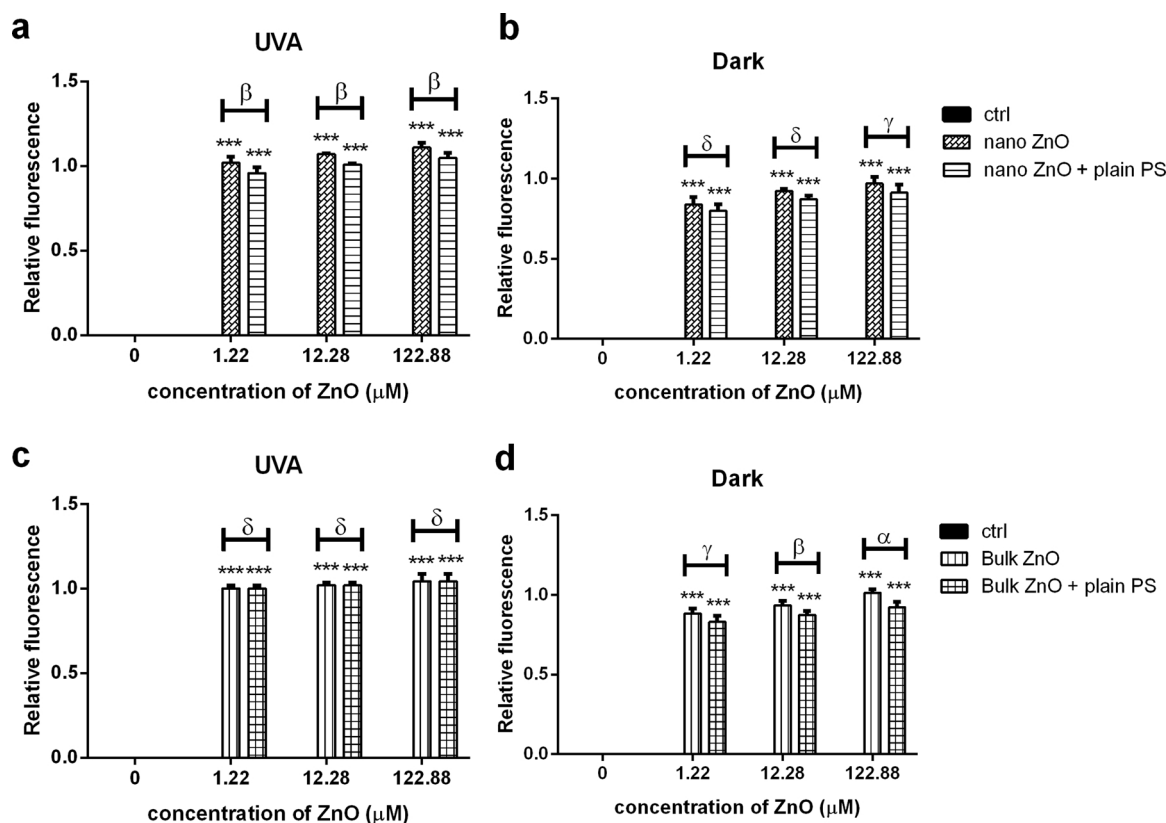


Fig. 4. Percentage of extracellular ROS generated from (a and b) nano-sized ZnO particle and (c and d) bulk ZnO particle under UV–A and dark conditions in the absence and presence of plain PS microplastics ($n = 3$). *** ($p < 0.001$) represented the significance between individual nano ZnO and bulk ZnO particle with respect to control group; whereas α , β , γ and δ represented the significance between the nano ZnO and bulk ZnO particles in the presence and absence of plain PS microplastics. α ($p < 0.001$), β ($p < 0.01$), γ ($p < 0.05$), and δ (ns).

intracellular reactive oxygen species production was noticed in both bulk and nano-sized ZnO particles for all the test concentrations. This effect was more pronounced under UV-A than dark conditions ($p < 0.05$) at all the test concentrations.

3.5.3. Evaluation of lipid peroxidation

The percentage of LPO produced by bulk and nano-sized ZnO particles individually and in combination with plain PS microplastics is graphically represented in (Fig. 6). The increment in the LPO production was observed with increasing concentrations of both bulk and nano-sized ZnO particles both under UV-A and dark conditions. The difference in LPO production was statistically significant ($p < 0.05$) with respect to that of control for all test concentrations. LPO production was increased in the case of nano-sized ZnO compared to bulk-ZnO under UV-A and dark conditions.

When plain PS microplastics were combined with bulk and nano-sized ZnO particles, a notable decline in LPO production was observed for all test concentrations. The decline in the LPO production was noticed to be enhanced under UV-A than dark conditions ($p < 0.05$) at all the test concentrations.

4. Discussion

The colloidal stability of bulk and nano-sized ZnO particles in ASW was evaluated as this plays a significant role in deciding the toxic effects [31]. The size of the particles increased to the micron range within 24 h (Fig. 2). Not only this, but Brownian motion of the particles in suspension may lead to enhanced interaction between particles, and consequently they may aggregate [32]. The negatively charged *Dunaliella salina* (-4.5 ± 1.2 mV) might interact with positively charged bulk and nano-sized ZnO particles (7.3 ± 2.6 mV and 10.49 ± 2.48 mV)

resulting in hetero-aggregation (Figs. S3 and S4, Supplementary information) between algae and particles. This entrapment of the algal cells in the aggregates could contribute to the toxic effects of the particles [28]. Large-sized aggregates of nano ZnO particles leading to the entrapment of the algal cells were also mentioned in other studies [33]. In the presence of plain PS microplastics, heteroaggregates formation was considerably reduced, possibly lessening the algal entrapment (Figs. S3 and S4, Supplementary Information), which could lead to a reduction in toxicity effects.

A decline in cell viability was noted for both individual bulk ZnO particles and ZnO nanoparticles, correlating with an increase in test concentrations under UV-A and dark condition (Fig. 3). [33,12,34] also noticed that the effects were less pronounced for bulk ZnO particles, and points to nano-sized effects in their previous studies. Numerous small-sized loosely packed cellular flocs confirmed hetero aggregation between the cells and nanoparticles (Figs. S3 and S4, Supplementary information). The mechanical damage caused by these aggregates may have contributed to the toxic effects of the zinc oxide nanoparticles and is evidenced by [33,12]. Sjollem [16] reported that microalga growth was inhibited by plain polystyrene particles only at exceedingly high concentrations (250 mg/L). In the presence of plain PS microplastics, a dose-dependent increase in cell viability was noted for bulk and nano-sized ZnO particles (Fig. 3). The decreased toxic effects in the presence of microplastics may be attributed to reduced heteroaggregation in the medium. The uncharged plain PS microplastics surrounding the algal cells may act as a barrier preventing the interaction between the negatively charged algal cells and positively charged nanoparticles, and thus limit the formation of heteroaggregates (Figs. S3 and S4, Supplementary information). Further studies are required to look into the different modes of interaction between the nano ZnO particles, microplastics, and the algal cells in the ASW medium. The toxicity of

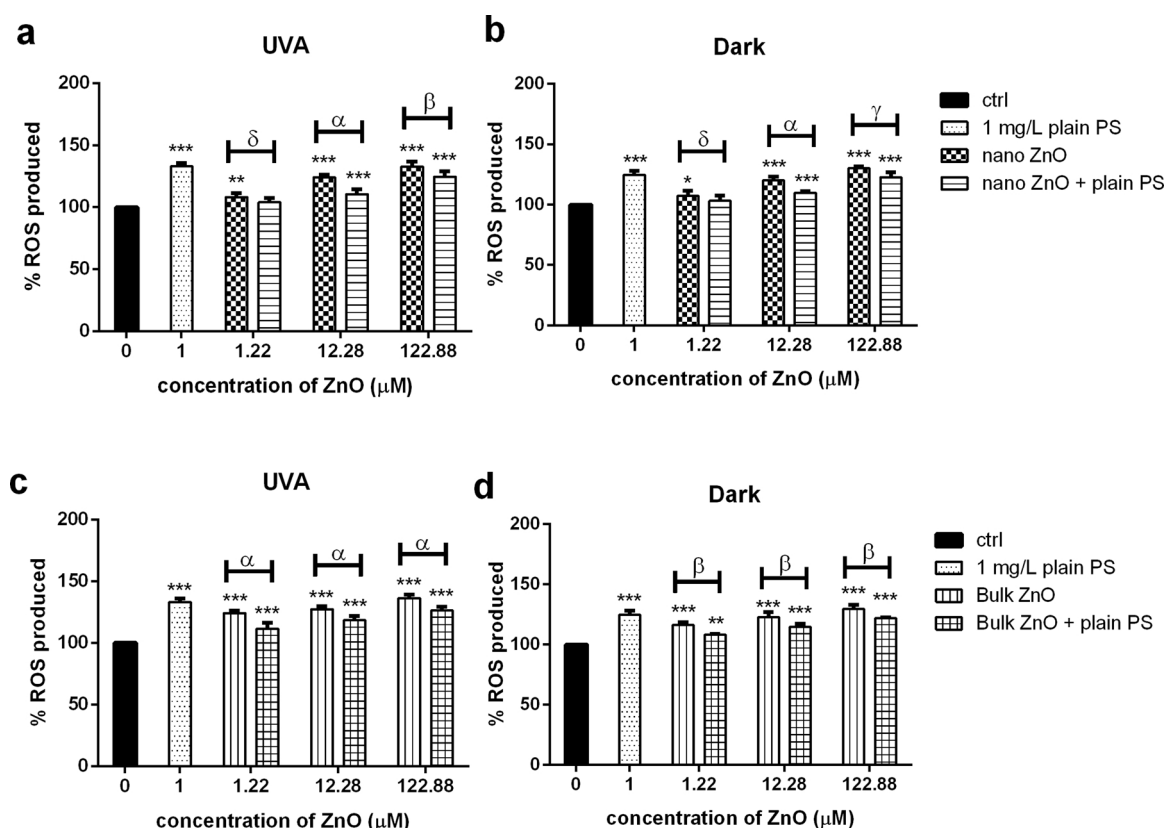


Fig. 5. Percentage of intracellular ROS generated by (a and b) nano-sized ZnO particle and (c and d) bulk ZnO particle in the absence and presence of plain PS microplastics under UV-A and dark condition ($n = 3$). *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$) denoted the significance between individual nano ZnO and bulk ZnO particle with respect to control samples whereas α ($p < 0.001$), β ($p < 0.01$), γ ($p < 0.05$), and δ (ns) represented the significance between the nano ZnO and bulk ZnO particles in the presence and absence of plain PS microplastics.

dissolved ions released from ZnSO_4 was found to be less when compared to that of nano-sized ZnO particles irrespective of the presence of plain PS microplastics under UV-A and dark conditions (Figs. S5 and S6, Supplementary information). Similar results were obtained in a study by Lee [7], who reported on the toxicity potential of nano ZnO particles from the dissolution of ions towards green algae.

An increase in extracellular ROS with increasing concentration was observed for both bulk and nano-sized ZnO particles. The extracellular ROS production was enhanced under UV-A conditions for nano-sized ZnO particles (Fig. 4), and may interact with the algal cell membrane, and lead to increased membrane permeability of *P. subcapitata*, [35]. On the contrary, when plain PS microplastics were added to bulk and nano-sized ZnO particles, a concentration-dependent decrease in the extracellular ROS was noted (Fig. 4). The abiotic ROS generation can be related to surface reactivity of the particles, and the presence of the microplastics could have attenuated the reactivity of the particles. The change in the surface reactivity through NP-microplastics interactions needs to be addressed in future studies. The reduction in abiotic ROS production in the presence of microplastics can be linked to the decrease in the toxic effects of these particles.

Similarly, a concentration-dependent increase in the intracellular ROS production was observed in *Dunaliella* sp. upon the interaction with bulk and nano-sized ZnO particles under UV-A and dark conditions. The smaller sized aggregates with high surface reactivity could cause mechanical damage to the cell membranes and facilitate the entry of particles. Intracellular ROS production was enhanced under UV-A compared to dark conditions. In the case of a mixture of plain PS microplastics with bulk and nano-sized ZnO particles, a decrement in the intracellular ROS generation was noted (Fig. 5). Both types of ZnO particles exerted reduced oxidative stress in the presence of microplastics, which again can be correlated to enhanced cell viability of the

test organisms

In the current study, the increase in LPO production with increasing concentration of bulk and nano-sized ZnO particles in *Dunaliella salina* indicates particle-induced damage to the lipid membrane. The production of LPO was enhanced under UV-A rather than dark conditions. Upon the addition of microplastics, a significant decline in LPO production was noted in *Dunaliella salina* under UV-A and dark conditions (Fig. 6). This reduction in lipid peroxidation in the presence of the microplastics can be ascribed to decreased abiotic ROS generation and reduced heteroaggregation of the particles in the medium as discussed in the preceding paragraphs. The reduced LPO production would necessarily mean less cell membrane damage in the algae, signifying a related decline in the uptake of the NPs. This could have reduced the intracellular ROS generation in presence of microplastics (Fig. 5).

5. Conclusion

The present work investigated the influence of uncharged plain PS microplastics on the toxic effects of bulk and nano-sized ZnO particles in the marine algae *Dunaliella salina*. The formation of loosely bound small-sized heteroaggregates through surface charge-based interaction with the algal cells contributed most towards the toxic effects of the nanoparticles. The presence of plain PS microplastics in the medium at low concentration (1 mg/L) decreased heteroaggregates formation, and thus the toxic effects too. The cushioning effects of microplastics on the toxic impact of ZnO particles onto the algae was also substantiated with decreased responses in different biomarkers, such as intracellular ROS generation and lipid peroxidation.

In the absence of previous reports on the interactive effects of plain PS microplastics and ZnO particles in marine algae, the current work will lead to more comprehensive studies in marine ecotoxicology on the

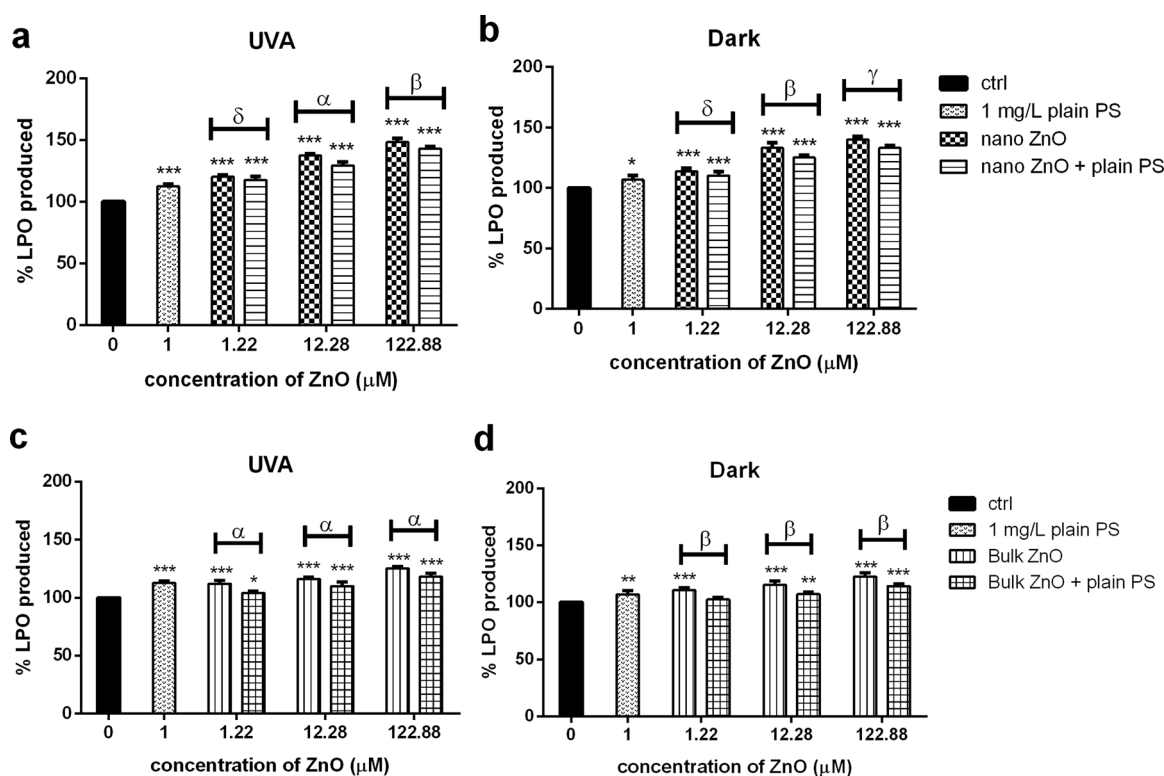


Fig. 6. Percentage of LPO produced by (a and b) nano-sized ZnO particle and (c, d) bulk ZnO particle in the absence and presence of plain PS microplastics under UV-A and dark conditions ($n = 3$). *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$) denoted the significance between individual nano ZnO and bulk ZnO particle with respect to control sample; whereas α ($p < 0.001$), β ($p < 0.01$), γ ($p < 0.05$), and δ (ns) represented the significance between the nano ZnO and bulk ZnO particles in the presence and absence of plain PS microplastics.

binary effects of emerging pollutants. The effects of surface change of the microplastics and the possible impact of ZnO dissolution in the mixture can be considered for future studies, to determine threshold conditions for modulating the toxicity of particles.

CRediT authorship contribution statement

Dhivya Gunasekaran: Investigation, Methodology, Visualization, Formal analysis, Writing - original draft. **Natarajan Chandrasekaran:** Formal analysis, Resources. **David Jenkins:** Writing - review & editing. **Amitava Mukherjee:** Conceptualization, Methodology, Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jece.2020.104250>.

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